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(54) Title: PROCESS FOR THE PREPARATION OF ANTIVIRAL PLANT TRANSFORMED WITH LACTOFERRIN GENE			
(57) Abstract			
<p>A process for the preparation of an antiviral plant, which comprises transforming a plant with an expression vector containing a lactoferrin(Lf) gene and culturing the transformed plant.</p> 			

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PROCESS FOR THE PREPARATION OF ANTIVIRAL PLANT  
TRANSFORMED WITH LACTOFERRIN GENE

FIELD OF THE INVENTION

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The present invention relates to an antiviral plant producing lactoferrin and process for the preparation thereof. More specifically, it pertains to an antiviral plant producing lactoferrin, which is transformed with a 10 lactoferrin(Lf) gene; a process for the preparation of the antiviral plant, which comprises transforming a plant with an expression vector containing a lactoferrin gene and culturing the transformed plant.

15 BACKGROUND OF THE INVENTION

Hitherto, various attempts have been made to develop a plant resistant to viral infection(hereinafter, an antiviral plant) by employing recombinant DNA technologies. For 20 example, Powell Abel et al. introduced a cDNA encoding the coat protein of a virus to a plant(Infect Immun., 35, 792-796(1986)), while the introduction of a defective viral replicase to a plant was attempted by Anderson et al. (P. N. A. S., 89, 8759-8763(1992)). However, these methods are not 25 practical in that, in order for a plant to have antiviral properties against a number of viruses, all the target viral genes must be introduced to the plant.

To solve this problem, Lodge et al. reported a method for conferring a general antiviral property on a plant by 30 introducing thereto a gene encoding an antiviral protein, e.g., RIP(ribosome inhibiting protein)(P. N. A. S., 90, 7089-7093(1993)). Although the plant transformed by this method shows an antiviral activity against various viruses, there exists the problem that the antiviral protein may be 35 harmful to human, and hence, the application of the above method may be restricted.

Lactoferrin is a conjugated protein found in mammals,

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e.g., in human milk, saliva, tear, semen and leukocyte. It affects the growth and differentiation of cells and has an antibacterial and immunopotentiative activity(Arold et al., Science, 197, 263-265(1977)). Further, it shows an 5 antiviral activity against Friend virus(Lu et al., Cancer Res., 47, 4184-4188(1987)), human immunodeficiency virus(HIV) and human cytomegalovirus(HCMV)(Meijer et al., J. Disease, 172, 380-388(1995)).

Mitra and Zhang reported a method for providing a plant 10 having an antibacterial activity by introducing thereto a cDNA encoding human lactoferrin(Plant Physiol., 106, 977-981(1994)). They confirmed the production of human lactoferrin in a tobacco cell by conducting a western blotting analysis of a tobacco cell transformed with human 15 lactoferrin cDNA and anti-human lactoferrin antibody. They also reported that the proliferation of bacteria was significantly suppressed when the extract of the transformed tobacco cell was added to a culture of bacteria, e.g., Pseudomonas sp., which is pathogenic to tobacco. However, 20 Mitra and Zhang did not, or failed to produce a transformed tobacco plant from the transformed tobacco cells. Moreover, human lactoferrin produced in the transformed tobacco cell was defective, i.e., significant parts thereof were missing, while the antibacterial activity was confirmed only in 25 vitro. Accordingly, the question of whether or not an antiviral plant can be produced from a transformed tobacco cell was left unanswered in the prior art.

#### SUMMARY OF THE INVENTION

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Accordingly, it is an object of the present invention to provide an antiviral plant producing lactoferrin.

Another object of the present invention is to provide a process for preparing said antiviral plant.

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A further object of the present invention is to provide a process for producing lactoferrin in a plant.

In accordance with one aspect of the present invention,

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there is provided a process for preparing an antiviral plant producing lactoferrin, which comprises transforming a plant with an expression vector containing lactoferrin(Lf) gene.

5 BRIEF DESCRIPTION OF THE DRAWINGS

The above and other objects and features of the present invention will become apparent from the following description of the invention, when taken in conjunction with 10 the accompanying drawings, in which:

Fig. 1 shows the structure of plasmid pLSM1 comprising human lactoferrin(HLf) gene;

Fig. 2 depicts the result of restriction enzyme analysis of plasmids pGA748 and pLSM1;

15 Fig. 3 displays the result of PCR analysis confirming the presence of HLf gene in the transformed tobacco shoots;

Fig. 4 provides the result of southern blot analysis confirming the presence of HLf gene in the transformed tobacco plants;

20 Fig. 5 presents the result of northern blot analysis confirming the expression of HLf gene in the transformed tobacco plants;

Fig. 6 exhibits the result of immuno blot analysis confirming the expression of HLf gene in the transformed 25 tobacco plants;

Fig. 7 shows the antiviral property of transformed tobacco plants after 4 weeks from viral infection; and

Fig. 8 illustrates the antiviral property of the first generation progenies of the transformed tobacco plants.

30

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides an antiviral plant producing lactoferrin and a process for preparing the 35 antiviral plant, which comprises transforming a plant with an expression vector containing a lactoferrin(Lf) gene and culturing the transformed plant using a suitable medium.

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The lactoferrin gene of the present invention may be any one of the mammalian lactoferrin genes, preferably, human lactoferrin gene(HLf). The lactoferrin gene may be prepared by a conventional chemical DNA synthesis method 5 using the known nucleotide sequence of the lactoferrin gene. For instance, HLf gene may be chemically synthesized using the nucleotide sequence of HLf gene disclosed by, e.g., Powell and Ogden(Nucleic Acids Research, 18, 4013(1990)). The lactoferrin gene of the present invention may also be 10 obtained by digesting with suitable restriction enzymes a plasmid comprising the gene and, in case of human lactoferrin gene, it may be obtained by digesting with suitable restriction enzymes a plasmid, e.g., plasmid pBHL-1 wherein the HLf gene is inserted in plasmid pBSK(Yong-Yeon 15 Cho, Cloning of Human Lactoferrin Gene and Its Polymorphism in Normal and Cancer Cells, August, 1993, a master's thesis in Science, Biology dept., Chungnam Univ., Korea). Any nucleotide substitution, deletion or addition in the nucleotide sequence of the lactoferrin gene may be made 20 provided that the property and function thereof remains substantially unchanged.

On the other hand, an expression vector comprising the lactoferrin gene may be prepared in accordance with a conventional DNA manipulation technique, e.g., by inserting 25 the gene to a suitable vector which may be selected from known vectors based on its compatibility with the intended host plant cell. For instance, the HLf gene prepared by digesting plasmid pBHL-1 with HindIII and BamHI may be inserted into the HindIII/BglII digestion site of a plant 30 vector containing controllable CaMV(cauliflower mosaic virus) 35S promoter, e.g., plasmid pGA748(Pap. Am. Chem. Soc., 205(1993)), to obtain plasmid pLSM1.

An Agrobacterium sp. cell may then be transformed with the expression vector prepared above in accordance with a 35 conventional method, e.g., the freeze-thaw method of Holsters et al. (Mol. Gen. Genet., 163, 181-187(1978)). The transformed Agrobacterium may in turn be used to transform

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a plant cell by employing, e.g., the Agrobacterium-mediated transformation method described by Robert, B. H., et al. (Plant Molecular Biology Manual, A42, 1-9(1988)).

Then, the resulting transformed plant cell may be  
5 cultured using a suitable medium, e.g., the MS(Murashige and Skoog) medium(Physiol. Plant, 15, 473-497(1962)) which contains BAP(6-benzylaminopurine), NAA(naphthaleneacetic acid) and kanamycin. This medium induces shoot growth from the transformed plant cell. Then, the induced shoots may be  
10 further cultured using the MS medium containing kanamycin to induce roots. The resulting transformed plant shoot having a root may be transplanted in soil to obtain a complete plant having resistance against viral infection.

Suitable plants for use in the present invention  
15 include plants belonging to the family Solanaceae, e.g., a tobacco plant.

The antiviral plant prepared in the present invention produces an intact lactoferrin and shows an antiviral activity against the cucumovirus, e.g., CMV(cucumber mosaic  
20 virus).

Accordingly, the present invention also provides a process for producing lactoferrin in a plant, which comprises culturing a plant transformed with an expression vector containing a lactoferrin gene.

25 For instance, human lactoferrin may be mass-produced by culturing a plant transformed with an expression vector containing human lactoferrin gene, e.g., plasmid pLSM1.

The following Examples are intended to further illustrate the present invention without limiting its  
30 scope.

Further, percentages given below for solid in solid mixture, liquid in liquid, and solid in liquid are on a wt/wt, vol/vol and wt/vol basis, respectively, unless specifically indicated otherwise.

Example 1: Preparation of Transformed Tobacco Producing Human Lactoferrin

(Step 1)

5

A human lactoferrin(HLf) gene containing 3' and 5' UTRs(untranslated regions)(GenBank accession No.: U07642), which was isolated from human mammary gland tissue cDNA library(Clontech, U.S.A.), was subcloned into the EcoRI site 10 of plasmid pBluescript II SK(pBSK, Promega, U.S.A.) to construct a plasmid designated pBHL-1(Yong-Yeon Cho, supra).

Plasmid pBHL-1 was digested with restriction enzymes HindIII and BamHI to obtain a 2.4 kb HLf gene. The HLf gene was inserted into the HindIII/BglII digestion site of 15 plasmid pGA748(Pap. Am. Chem. Soc., 205(1993)), said digestion site being downstream from the CaMV(cauliflower mosaic virus) 35S promoter site. The resulting plasmid was designated plasmid pLSM1 and its structure is shown in Fig. 1, wherein NPTII represents kanamycin resistant gene; 35S, 20 CaMV 35S promoter; and HLf, human lactoferrin gene.

E. coli DH5 $\alpha$ (Clontech) was transformed with plasmid pLSM1 in accordance with the CaCl<sub>2</sub> method(Cohen et al., P. N. A. S., 69, 2110(1972)). E. coli DH5 $\alpha$  transformed with plasmid pLSM1 was deposited on May 10, 1995 with the Korean 25 Collection for Type Cultures(KCTC)(Address: GERIC, KIST, P.O. Box 115, Yusong, Taejon, 305-600, Republic of Korea) under the accession number of KCTC 0159BP in accordance with the terms of Budapest Treaty on the International Recognition of the Deposit of Microorganism for the Purpose of Patent 30 Procedure.

The result of restriction enzyme analyses is shown in Fig. 2, wherein a 11.6 kb DNA fragment is observed when plasmid pGA748 is digested with EcoRI and HindIII(lane 1); a 11.8 kb DNA fragment and additional 2.2 kb DNA are 35 observed when plasmid pLSM1 is digested with EcoRI and HindIII(lane 2); and, when plasmid pLSM1 is digested with BglIII, which has a restriction site in the HLf gene segment,

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13.4 kb and 0.6 kb DNA fragments are observed(lane 3) as expected from the restriction enzyme map. Lane M shows a standard DNA size marker.

5 (Step 2)

Plasmid pLSM1 was introduced to Agrobacterium LBA4404(Clontech, U.S.A.) in accordance with the freeze-thaw method of Holsters et al.(Mol. Gen. Genet., 163, 10 181-187(1978)). The resulting transformed Agrobacterium LBA4404 was cultured together with leaf disks of a tobacco plant, Nicotiana tabacum L. Wisconsin 38, in order to transform the tobacco leaves with HLf gene, in accordance with the combination culture method(Horsch et al., Science, 15 227, 1229-1231(1985)).

The transformed leaf disks were cultured in a shoot-inducing medium, i.e., MS(Murashige and Skoog) medium (Physiol. Plant., 15, 473-497(1962)) containing 0.1 mg/l of BAP(6-benzylaminopurine), 1.0 mg/l of NAA(naphthaleneacetic acid), and 150 µg/ml of kanamycin. Selected shoots resistant to kanamycin were further cultured in the MS medium containing 200 µg/ml of kanamycin to obtain tobacco plants having roots.

25 (Step 3)

To investigate whether the tobacco shoots prepared above comprise HLf gene, polymerase chain reactions(PCR) were carried out in accordance with the method described by 30 Edward et al.(Nucleic Acids Res., 19, 1349(1991)), wherein used are 100 mg each of the tobacco leaf disks and primers 1(5'-AGTGGCTTGAGCGAAGG-3') and 2(5'-GGCCGCGGTTTACTCCTGAG-G-3') prepared based on the nucleotide sequence of the HLf gene described by Powell and Ogden(supra). The result is 35 shown in Fig. 3.

Observable in Fig. 3 are 1 kb-sized PCR bands associated with 9 out of the 12 plants tested, wherein lanes

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1 to 12 represent the transformed tobacco plants; C, an untransformed tobacco plant; +, plasmid pLSM1; and M, standard DNA size markers.

5 (Step 4)

The transformants confirmed in (Step 3) were transplanted in soil and grown at 25°C for 2 to 4 weeks under a 16-light hours/day condition. A 1 g leaf sample of 10 each of the plants was ground and the genomic DNA was separated therefrom in accordance with the modified Shure's method(Cell, 35, 225-233(1985)). Total RNA was also separated from the genomic DNA in accordance with the modified Sambrook's method(Molecular Cloning: A Laboratory Manual, 2nd Ed., pp 7.19-7.22, 1989, Cold Spring Harbor Laboratory Press, New York, U.S.A.) using CsCl.

15 10 µg each of the genomic DNA from each tobacco plant was digested with HindIII and subjected to a electrophoresis on 0.8% agarose gel. 40 µg each of the total RNA from each 20 tobacco plant was electrophoresed on 1% agarose gel containing formaldehyde.

Each of the genomic DNAs and the total RNAs separated on the agarose gel was transferred to Nytran membrane (Schleicher & Schuell, Germany) and subjected to southern 25 and northern blot analyses by using <sup>32</sup>P-labelled HLF cDNA as a probe. It was confirmed by the southern blot analysis result that HLF gene was inserted in the genomic DNA of tobacco(Fig. 4). In Fig. 4, lanes 1 to 14 correspond to the transformants and Co, the untransformed tobacco. It was 30 also confirmed by the northern blot analysis result that HLF gene was expressed in the transformants; various amount of 2.3 kb transcripts were observed in 8 out of the total 9 transformants(Fig. 5). In Fig. 5, lanes 1 to 9 correspond to the transformants and Co, the untransformed tobacco.

## (Step 5)

A 100 mg leaf sample of selected transformed tobacco plants was ground and suspended in phosphate buffered saline(PBS) containing 1 mM phenylmethylsulfonyl fluoride(PMSF). The resulting suspension was centrifuged at 12,000 rpm for 10 min. to obtain a supernatant containing water-soluble proteins.

50 µg or 100 µg each of the water-soluble proteins was subjected to 10% SDS-PAGE by employing Laemmli's method (Nature 227, 680(1970)). The proteins separated by size on the gel were transferred onto a nitrocellulose membrane (Hoefer) by employing an electrophoretic transfrerer (Bio-Rad). The membrane was put into Tris-buffered saline (TBS) containing 3% bovine serum albumin(BSA) to block the unbound portion of the membrane. 300 ng of anti-HLf antibody(Sigma) in TBS containing 1% bovine serum albumin (BSA) was added to the membrane and reacted at room temperature for 2 hours to allow the binding of protein with the antibody.

The membrane was then washed with TBS, placed in an anti-human antibody solution, which was prepared by diluting goat anti-human IgG labeled with alkaline phosphatase(Sigma Co., U.S.A.) with 10,000-fold volume of TBS containing 1% BSA, and reacted at room temperature for 1.5 hour. The membrane was washed thoroughly with TBS and added thereto was 10 ml of BCIP/NBT(5-bromo-4-chloro-3-indolylphosphate/nitro blue tetrozorium) solution. The resultant was reacted at room temperature for 10 min. to develop a color reaction.

30 The result in Fig. 6 shows that all transformants produced lactoferrin, while the untransformed tobacco did not. In Fig. 6, lanes 1 to 3 represent the result obtained with 100 µg protein samples, and lanes 4 to 6, 50 µg protein samples. Further, lanes 1 and 4 correspond to the untransformed tobacco and lanes 2, 3, 5 and 6, the transformed tobacco. The transformed tobacco plants produce not only a 80 kDa protein which is identical in size with

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human lactoferrin(Sigma) but also 38 or 42 kDa lactoferrin fragments.

Example 2: Antiviral Activity of the Transformants

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(Step 1) First Viral Infection with CMV-Y Virus

Three tobacco plants induced from untransformed leaf disk, one tobacco plant transformed with pGA748, and three 10 tobacco plants transformed with pLSM1 were transplanted to soil and grown under the same condition as in (Step 4) of Example 1.

After 4 weeks from the transplantation, one new leaf from each plant was scratched with a carborundum powder, 15 infected along a vein with a tobacco leaf suspension containing CMV-Y virus(J. Gen. Virol., 74(1), 319-322 (1993)), and, then, washed with water to remove the carborundum powder. One of the untransformed plants developed disease symptoms such as chlorosis, necrosis and 20 systemic infection after 7 days from the viral infection, while all the other tobacco plants were healthy over 4 weeks after the viral infection.

(Step 2) Second Viral Infection with CMV-Y Virus

25

The six tobacco plants which remained healthy in (Step 1) were subjected to a second viral infection with 10 µg/ml of CMV-Y virus in accordance with the same procedure as in (Step 1). After 3 days from the infection, additional 30 disease symptoms were observed in one of the two untransformed tobacco plants as well as in the tobacco plant transformed with plasmid pGA748, while one remaining untransformed tobacco plant and the three tobacco plants transformed with plasmid pLSM1 were healthy even after 2 35 weeks from the second infection.

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(Step 3) Third Viral Infection with CMV-Y Virus

One untransformed tobacco plant and three tobacco plants transformed with pLSM1, which remained healthy in  
5 (Step 2) were subjected to a third viral infection with 10 µg/ml of CMV-Y virus in accordance with the same procedure as in (Step 1). After 3 days from the infection, additional disease symptoms were observed in the untransformed tobacco plant, while three tobacco plants transformed with pLSM1  
10 were healthy even after 2 weeks from the third infection and their growth rate were similar to those of uninfected tobacco plants(Fig. 7). In Fig. 7, 1 represents the untransformed tobacco plant; 2, tobacco plant transformed with plasmid pGA748; 3, tobacco plant transformed with  
15 plasmid pLSM1.

Example 3: Antiviral Activity of the First Generation  
Progeny of the Transformant

20 The tobacco plants transformed with plasmid pLSM1 or pGA748 were self-pollinated to obtain seeds, and then, the transformants growing on MS medium containing 150 µg/ml kanamycin were selected. Those tobacco plants, which were transformed with plasmid pLSM1 and considered to have 1 copy  
25 of HLf gene shown in Fig. 4, lines 1 and 3, produced kanamycin resistant and kanamycin susceptible progenies in ratios of 140:48 and 90:30, respectively. This result shows that the antiviral character is inherited in a ratio of 3:1 in accordance with Mendelism.

30 The kanamycin resistant progenies were transplanted in soil, grown at 25°C for 2 to 4 weeks under a 16-light hours/day condition, and then, subjected to a viral infection in accordance with the same procedure as in (Step 6) of Example 1. The tobacco plants transformed with pGA748  
35 showed disease symptoms at the third day from the infection. However, the tobacco plants transformed with pLSM1 exhibited localized, mild symptoms only on the infected leaf and a

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systemic infection was not observed even after 4 weeks from the infection. Further, they showed a growth rate similar to that of uninfected tobacco plants. The result is shown in Fig. 8, wherein 1 is a tobacco plant transformed with 5 plasmid pGA748; 2, a tobacco plant transformed with plasmid pLSM1; and Co, an uninfected tobacco plant transformed with plasmid pGA748.

Example 4: Preparation of Callus from Transformed Tobacco  
10                   Plant

A callus was induced from the pLSM1-transformed tobacco progeny plant of Example 3 in accordance with the method of J. Draper et al. (Plant Genetic Transformation and Gene Expression, Chap. 2, 1988, Blackwell).  
15

Specifically, a leaf sample from the tobacco progeny plant transformed with plasmid pLSM1 was treated with 0.5-1.0 % of hypochlorite and washed several times with the sterilized water to remove the hypochlorite.  
20

On the other hand, 2.0 mg/l of NAA and 0.5 mg/l of BAP were added to the MS medium and the mixture was autoclaved. 150 mg/l of kanamycin was added to the sterilized medium and aliquots of the resulting medium were placed in Petri dishes and solidified.  
25

The disinfected leaf sample prepared above was cut by 0.5 cm x 1 cm size and placed on the MS medium containing NAA, BAP and kanamycin in a Petri dish. The Petri dish was sealed and maintained at 25°C in the dark. Two weeks after, undifferentiation of the leaf was observed; i.e., a callus 30 was formed at the edge of the leaf pieces.  
30

The callus was preserved on the same medium with subculturing monthly.

A callus of the tobacco plant transformed with plasmid pLSM1, i.e., Nicotiana tabacum W38(pLSM1) was deposited on 35 May 16, 1996 with the Korea Research Institute of Bioscience and Biotechnology, Korean Collection for Type Cultures(KCTC) (Address: KCTC, KRIBB, #52, Oun-dong, Yusong-ku, Taejon,

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305-333, Republic of Korea) under the accession number of KCTC 0248BP in accordance with the terms of Budapest Treaty on the International Recognition of the Deposit of Microorganism for the Purpose of Patent Procedure.

5 An antiviral tobacco plant may be derived from the above callus by inducing shoot and root therefrom in accordance with the procedure of (Step 2) of Example 1, and then culturing the resulting plantlets in soil to develop into a whole plant(J. Draper et al., supra).

10

While the invention has been described with respect to the above specific embodiments, it should be recognized that various modifications and changes may be made to the invention by those skilled in the art which also fall within 15 the scope of the invention as defined by the appended claims.

What is claimed is:

1. A process for preparing an antiviral plant producing lactoferrin which comprises transforming a plant 5 with an expression vector containing a lactoferrin(Lf) gene.

2. The process of claim 1, wherein the plant belongs to the family Solanaceae.

10 3. The process of claim 2, wherein the plant is a tobacco plant.

4. The process of claim 1, wherein the expression vector is plasmid pLSM1(KCTC 0159BP).

15

5. The process of claim 1, wherein the antiviral activity is against cucumovirus.

20 6. The process of claim 5, wherein the cucumovirus is cucumber mosaic virus.

7. The process of claim 1, wherein the lactoferrin gene is human lactoferrin gene.

25

8. An antiviral plant producing lactoferrin, which is prepared by transforming a plant with an expression vector containing a lactoferrin gene.

30

9. The antiviral plant of claim 8, which belongs to the family Solanaceae.

10. The antiviral plant of claim 8, wherein the lactoferrin is human lactoferrin.

35

11. A process for producing lactoferrin in a plant, which comprises culturing a plant transformed with an expression vector containing a lactoferrin gene.

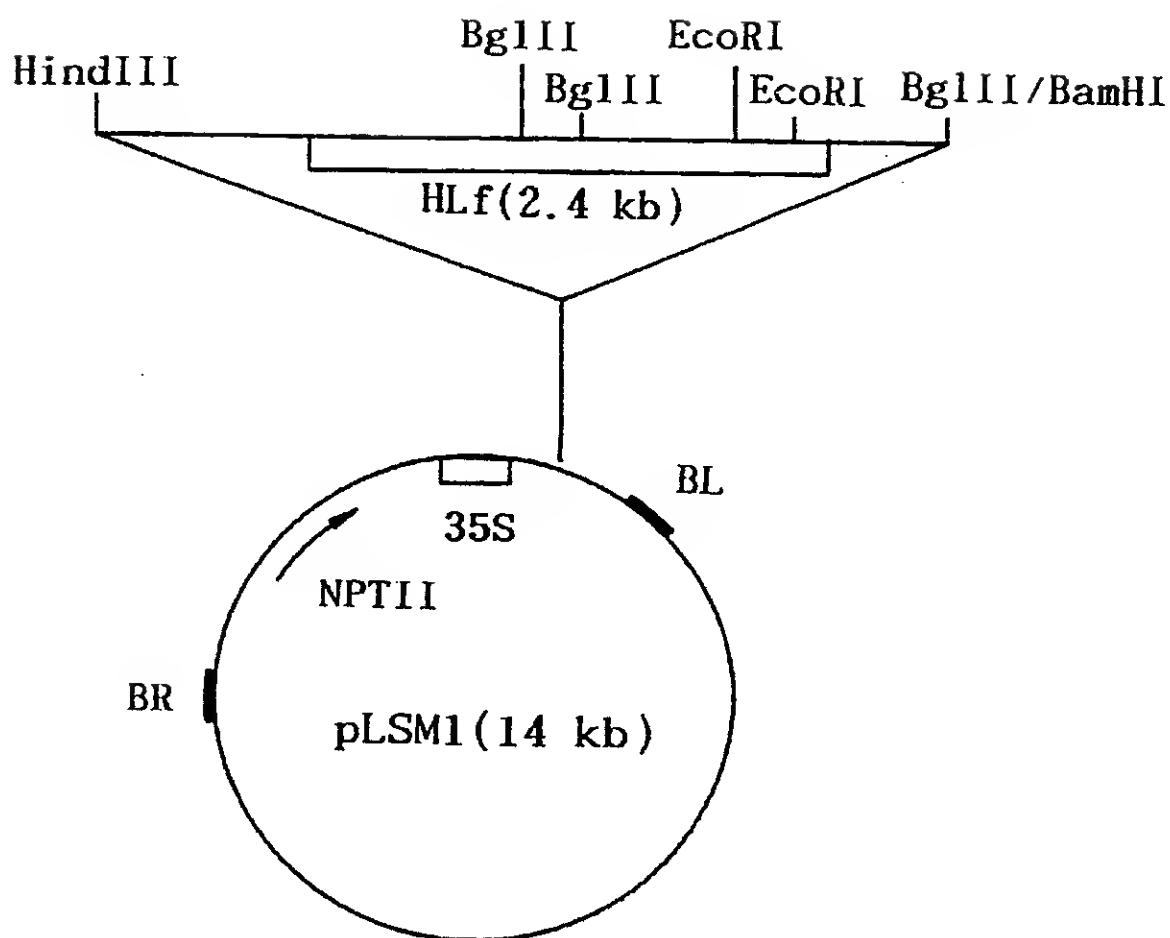
- 15 -

12. The process of claim 11, wherein the plant belongs to the family Solanaceae.

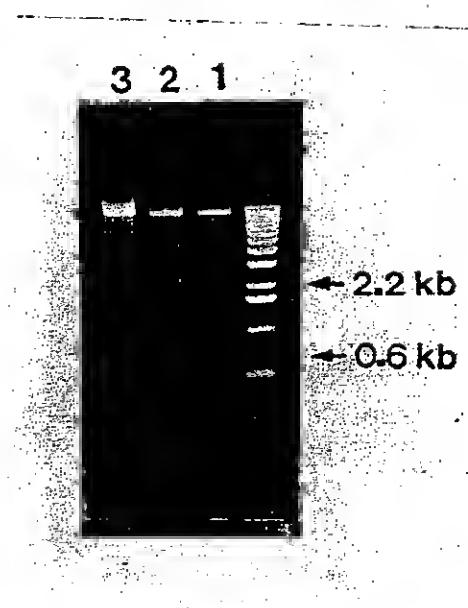
13. The process of claim 11, wherein the lactoferrin gene is human lactoferrin gene.

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Fig. 1



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**Fig. 2**

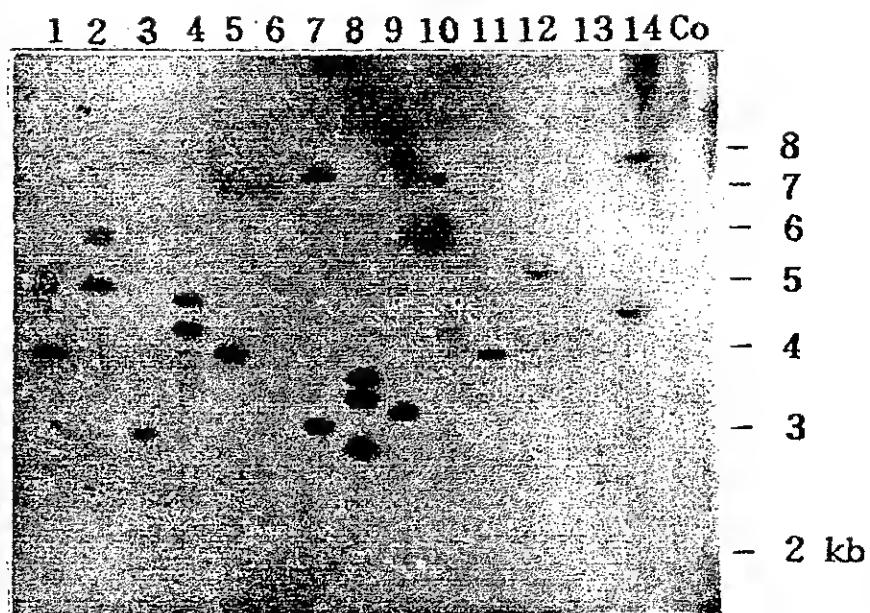


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Fig. 3

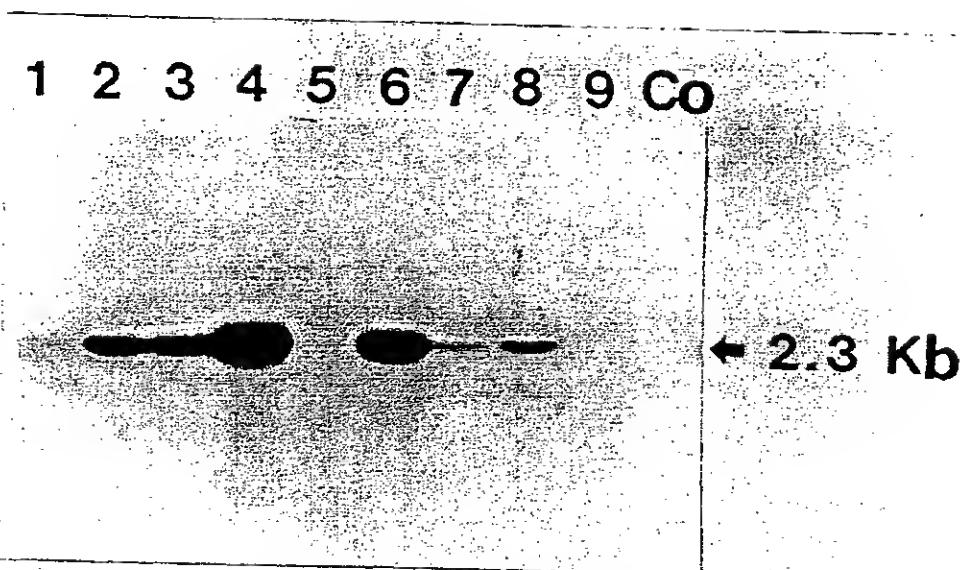


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**Fig. 4**



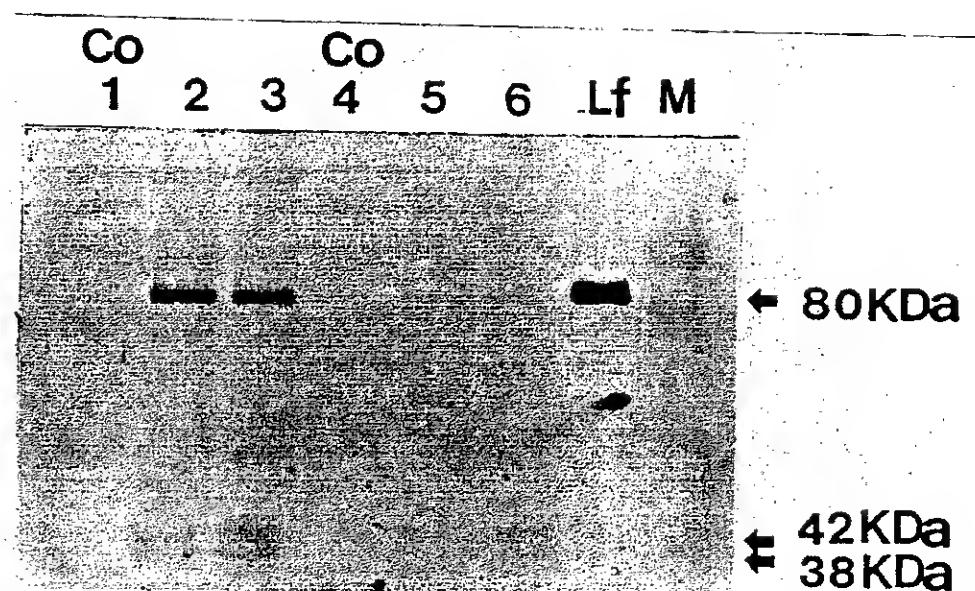
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Fig. 5

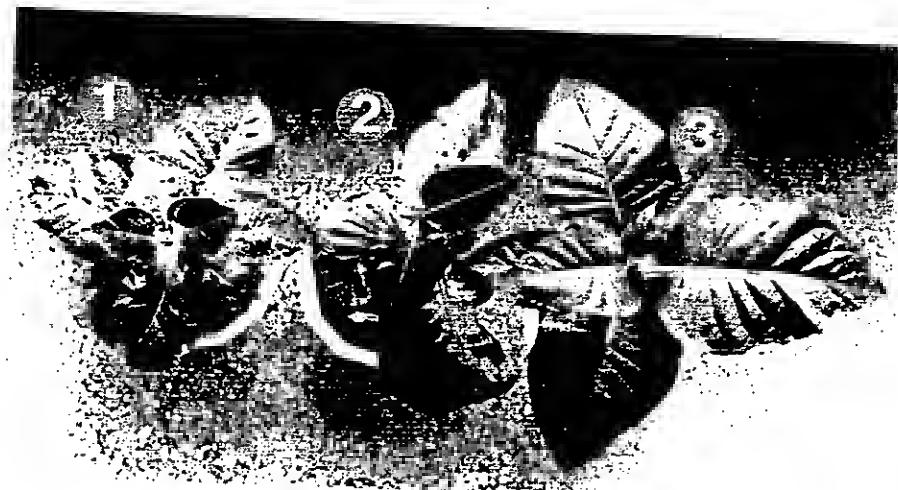


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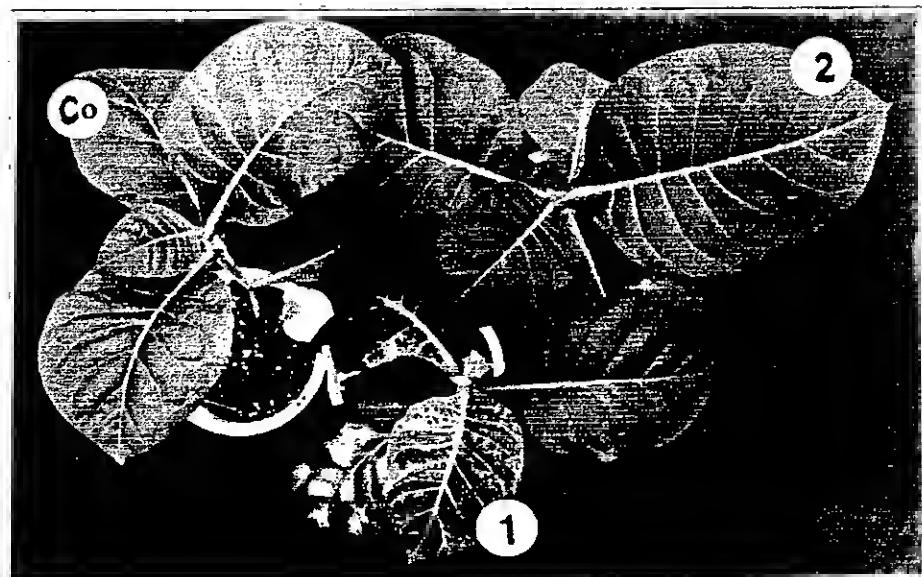
Fig. 6



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Fig. 7



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**Fig. 8**



## INTERNATIONAL SEARCH REPORT

International application No.

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## A. CLASSIFICATION OF SUBJECT MATTER

IPC<sup>6</sup>: A 01 H 1/00; C 12 N 15/83

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC<sup>6</sup>: A 01 H 1/00, 5/00; C 12 N 15/00, 15/83

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

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## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP 0 279 433 A2 (TAKEDA CHEMICAL INDUSTRIES LTD.) 24 August 1988 (24.08.88), abstract; column 1, lines 1ff.	1-3, 5, 6
A	EP 0 140 504 A1 (STAUFFER CHEMICAL COMPANY) 08 May 1985 (08.05.85), page 4, lines 27ff.	4

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**INTERNATIONAL SEARCH REPORT**  
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